I. Remarks

Claims 1-23, 27, 28, 38 and 39 have been canceled without prejudice or disclaimer. Applicants reserve the right to file one or more continuation applications directed to the subject matter encompassed by the canceled claims. Claims 24-26 and 35-37 have been amended. The amendments to the claims are supported by the specification as filed, for example, at page 223, lines 14-30. Upon entry of the present amendments, claims 24-26, 29-37, and 40-45 will be pending. No new matter has been added.

II. Objections to the Specification and Claims

The specification has been objected to because the Examiner alleges that documents have been improperly incorporated by reference. *See* present Office Action, mailed January 21, 2005, pages 2-4, part 2. More particularly, the Examiner states that, "[s]uch omnibus language fails to specify what specific information Applicant seeks to incorporate by reference and similarly fails to teach with detailed particularity just where that specific information is to be found in each of the cited documents." *See* present Office Action, page 3. Applicants respectfully disagree.

As a preliminary matter Applicants point out that the M.P.E.P. distinguishes between different kinds of information that can be incorporated by reference. For example, incorporation by reference of U.S. patent applications relied on only to establish an earlier effective filing date differs from incorporation by reference of other documents. More specifically, with respect to priority applications, the M.P.E.P. teaches that "[a]s a safeguard against the omission of a portion of a prior application for which priority is claimed under 35 U.S.C. 119(a)-(d) or (f), or for which benefit is claimed under 35 U.S.C. 119(e) or 120, applicant may include a statement at the time of filing of the later application incorporating by reference the prior application..." *See* M.P.E.P. § 609(p)I.B at page 600-85.

Alternatively, when an Applicant wishes to incorporate by reference nonessential subject matter that is not being relied upon to establish an earlier effective filing date, for the purposes of "...indicating the background of the invention or illustrating the state of the art," the M.P.E.P. states that, "...the referencing application should include an identification of the referenced [nonessential subject matter]. Particular attention should be directed to specific portions of the referenced document where the subject matter being incorporated may be found." *See* M.P.E.P. § 609(p)I.A at page 600-83.

With regard to the last sentence of Applicants' incorporation by reference paragraph on page 829[1], Applicants respectfully submit that the referenced applications are being relied upon to establish an earlier effective filing date for the present application (while not the earliest claim to the benefit of priority, the present application is a continuation-in-part of PCT/US01/05614, filed February 21, 2001, which is an application claiming the benefit of priority under 35 U.S.C. § 119(e) of application nos. 60/184,836 and 60/193,170, filed February 24, 2000 and March 29, 2000, respectively). Accordingly, Applicants respectfully request that this aspect of the Examiner's objection be reconsidered and withdrawn.

With regard to the first sentence of Applicants' incorporation by reference paragraph on page 829, Applicants respectfully submit that the documents being incorporated by reference are nonessential references. More particularly, these references are general background materials that demonstrate the state of the art at the time of filing. Accordingly, in compliance with M.P.E.P. 608.01(p)I.A., since these document as a whole are pertinent to the state of the art, the present specification states that, "The entire disclosure of each document cited...is hereby incorporated by reference." *See* specification page 829, emphasis added. Accordingly, Applicants respectfully request that this aspect of the Examiner's objection be reconsidered and withdrawn.

With regard to the second sentence of Applicants' incorporation by reference paragraph on page 829, Applicants respectfully submit that the sequence listing and the corresponding computer readable form filed with the specification were incorporated by reference to ensure that this material would be available for incorporation by reference into future divisional or continuation applications. If, however, the Examiner continues to object to the second sentence of the paragraph on page 829, Applicants are willing to remove it by amendment.

The Examiner also states that, "[t]he title of the invention is not descriptive." *See* present Office Action, mailed January 21, 2005, page 4, part 4. Applicants have amended the title herein as requested by the Examiner.

The Examiner further states that, "[c]laim 19 is objected to because...[it] depends

[1] "The entire disclosure of each document cited (including patents, patent applications, journal articles, abstracts, laboratory manuals, books, or other disclosures) in the Background of the Invention, Detailed Description, and Examples is hereby incorporated herein by reference. Further, the hard copy of the sequence listing submitted herewith and the corresponding computer readable form are both incorporated herein by reference in their entireties. Additionally, the specifications and sequence listings of International Application No. PCT/US01/05614 filed February 21, 2001, and of U.S. Provisional Applications Serial Nos. 60/184,836 and 60/193,170 are all hereby incorporated by reference in their entirety." *See* present specification, page 829.

from non-elected claim 11...[it] encompasses non-elected embodiments." *See* Office Action, page 4, part 5. Applicants disagree that claim 19 encompasses non-elected embodiments, however solely in the interest of facilitating prosecution, Applicants have canceled claim 19.

In light of the arguments and amendments given above, Applicants respectfully request that the Examiner's objections to the specification be reconsidered and withdrawn.

III. Rejections under 35 U.S.C. § 112 (written description)

Claims 19 and 24-45 have been rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement because they allegedly contain subject matter which was not described "...in such as way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention." *See* present Office Action, paragraph bridging pages 4-5. More particularly, the Examiner states:

> A review of the disclosure finds but a single instance where SEQ ID NO: 344 is even made, and then said occurrence is to be located at page 223, last two lines, where "predicted epitopes" of SEQ ID NO: 344 are suggested. A review of the disclosure fails to identify where SEQ ID NO:344 has been correlated with any disease, including pancreatic cancer. Page 23, lines 14-15, teach gene 96 "is expressed primarily in pancreatic tumor and ulcerative colitis, and to a lesser extent in several tumors and normal tissue." A review of the disclosure find the term "pancreatic cancer" has been used at the following occasions: Page 610, lines 5 and 12; page 613, line 29; page 614, line 24; page 635, page 31; page 636, line 25. At no time is the term used in conjunction with SEQ ID NO: 344. *See* present Office Action, paragraph bridging pages 6-7.

Applicants respectfully disagree and traverse. As a preliminary matter, claim 19 has been canceled, therefore rejection with respect to this claim is moot.

With regard to the Examiner's allegations that, "A review of the disclosure finds but a single instance where SEQ ID NO: 344 is even made... A review of the disclosure fails to identify where SEQ ID NO:344 has been correlated with any disease, including pancreatic cancer... At no time is the term used in conjunction with SEQ ID NO: 344."

Applicants respectfully disagree and submit that the specification provides ample nexus between SEQ ID NO: 344 and pancreatic cancer. In particular, the specification teaches that, "Table 1 summarizes the information corresponding to each 'Gene No.' described above." *See*, Specification, pages 488, first sentence. In this regard, Table 1

teaches that SEQ ID NO:344 corresponds to Gene No.: 96. *See* present specification page 467, Table 1, row 2. Thus, the disclosure for Gene No. 96 (found on page 222, line 16 through page 224, line 19) is directly connected with SEQ ID NO:344 not only by its specific mention on page 223 (last two lines), but also by the fundamental association with Gene No:96 in Table 1. Therefore, the disclosure relating to pancreatic cancer provided in the description of Gene No. 96 at pages 222-224 (discussed further below) is also fundamentally associated with SEQ ID NO:344.

The Examiner alleges that "...the disclosure fails to identify where SEQ ID NO:344 has been correlated with any disease, including pancreatic cancer," and further points to several places in the present specification where the specific term "pancreatic cancer" appears but not as part of an explicit statement directed toward SEQ ID NO:344. Applicants respectfully submit that the section of the specification entitled "Features of Protein Encoded by Gene No: 96," while not reciting the exact phrase "pancreatic cancer," nevertheless correlates SEQ ID NO:344 with disease terms equally synonymous with pancreatic cancer, such as "pancreas tumor" and "proliferative disorders, such as pancreatic disorders" as shown below (emphasis added):

> This gene is expressed primarily in pancreas tumor and ulcerative colitis, and to a lesser extent in several tumors and normal tissues. Polynucleotides and polypeptides of the invention are useful as reagents for differential identification of the tissue(s) or cell type(s) present in a biological sample and for diagnosis of diseases and conditions which include, but are not limited to, metabolic, gastrointestinal, or proliferative disorders, such as pancreatic disorders, ulcerative colitis, tumors and food poisoning. Similarly, polypeptides and antibodies directed to these polypeptides are useful in providing immunological probes for differential identification of the tissue(s) or cell type(s)....Protein, as well as, antibodies directed against the protein may show utility as a tumor marker and/or immunotherapy targets for the above listed tissues. *See* specification page 223, lines 14 to 22 and page 224, lines 7 to 9.

With respect to independent claims 24 and 35, it is alleged that "...the specification fails to identify what a standard level of protein is." *See* Office Action, page 6, section 10. Applicants respectfully disagree. With regard to Gene No: 96 and SEQ ID NO:344, the present specification teaches that, "...expression of this gene at significantly higher or lower levels may be routinely detected in certain tissues or cell types...relative to the standard gene

expression level, i.e., the expression level in healthy tissue or bodily fluid from an individual not having the disorder." *See* present specification, page 223, lines 24-30 (emphasis added). Nevertheless, solely in the interest of facilitating prosecution, Applicants have deleted the term "standard" from claims 24 and 35.

It is further alleged that the specification fails to teach that the antibodies and antibody fragments recited in the claims 29 and 40 have been "...developed and found useful in practicing the claimed method." *See* Office Action, page 7, part 10. Applicants respectfully disagree. Antibodies do not have to be "developed and found useful in practicing the claimed method" in order for one of ordinary skill in the art to practice the claimed method. The description in the present specification is sufficient to teach one of ordinary skill in the art how to make the antibodies and fragments thereof, *e.g.*, human antibodies, polyclonal antibodies, chimeric antibodies, monoclonal antibodies, Fab fragments etc., that specifically bind a protein of interest, *e.g.*, SEQ ID NO:344, and how to use these antibodies to assay for the protein of interest in a biological sample, such as pancreatic tissue. *See* present specification, page 504, line 25 to page 519, line 10; page 532, line 14 to page 534, line 20; page 545, line 18 to page 550, line 2; page 546, lines 9-18; and page 721, line 11 to page 723, line 2.

With regard to claims 32-34 and 40-45, it is alleged that, "...the specification does not provide an adequate written description of the antibodies, much less claimed labeled versions of same..." *See* Office Action, page 7, part 11.

Applicants respectfully disagree, and point out that labeled antibodies are adequately described, for example at specification page 546, lines 14-18[1].

In this same section of the present Office Action it is further stated that, "[a]ssuming arguendo, that the antibodies have been adequately described...the specification is essentially silent as to what constitutes normal or standard levels of the protein in the myriad sample types encompassed by the claimed method." Applicants respectfully disagree.

However, solely in the interest of facilitating prosecution, Applicants have canceled claims 27, 28, 38 and 39, and amended claims 24-26 and 35-37 to indicate that biological samples being assayed are pancreatic biological samples, in the form of pancreatic tissue or

[1]As stated on page 546, lines 14-18 (emphasis added), "Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase; radioisotopes, such as iodine (125I, 121I), carbon (14C), sulfur (35S), tritium (3H), indium (112In), and technetium (99Tc); luminescent labels, such as luminol; and fluorescent labels, such as fluorescein and rhodamine, and biotin."

cells (as supported in the specification, *e.g.*, at page 223, lines 16-30 and page 567, lines 10-18). It is also alleged that, "...Applicant is attempting to satisfy the written description requirement of 35 U.S.C. § 112, first paragraph, through obviousness... [o]bviousness, however, cannot be relied upon for satisfaction of the written description requirement," *See* Office Action page 7, part 12. Applicants request clarification and respectfully submit that the present specification provides explicit written description for all pending claims. Hence, no attempt has been made to claim subject matter which has merely been rendered obvious.

With regard to claim 35, which recites "the full-length polypeptide encoded by the HRDFB85 cDNA contained in ATCC™ Deposit Number 209082," the Examiner expresses several concerns about whether the deposited HRDFB85 cDNA actually encodes a polypeptide. *See* Office Action, page 8, part 13. For example, the Examiner expresses a concern as to whether, "...the nucleic acid in the vector is in proper reading order..." Applicants respectfully submit that upon consideration of the information contained in Table 1, row 2, page 467 of the present specification, in combination with the description of Table 1 on pages 488-492[1], one of ordinary skill in the art would be convinced that the deposited cDNA clone does actually encode a polypeptide.

More specifically, as shown in Table 1, the HRDFB85 cDNA contained within ATCC™ Deposit Number 97977 contains nucleotides 23 to 1697 of the 1705 nucleotide SEQ ID NO:106, meaning that the HRDFB85 cDNA contained within ATCC™ Deposit Number 97977 is 1697 nucleotides – 23 nucleotides = 1674 nucleotides in length and encompasses the predicted start codon of SEQ ID NO:106 ("5' NT of Start Codon" = nucleotide 233 of SEQ ID NO:106) and the 602 nucleotides of SEQ ID NO:106 that follow, which gives a total of 603 nucleotides encoding the 201 amino acids shown in SEQ ID NO:344.

A further concern that the Examiner express at page 8, part 13 of the present Office

[1] More particularly, as stated on page 488, lines 1-18 (emphasis added):

"Table 1 summarizes the information corresponding to each "Gene No." described above. The nucleotide sequence identified as "NT SEQ ID NO:X" was assembled from partially homologous ("overlapping") sequences obtained from the "cDNA clone ID" identified in Table 1 and, in some cases, from additional related DNA clones. The overlapping sequences were assembled into a single contiguous sequence of high redundancy (usually three to five overlapping sequences at each nucleotide position), resulting in a final sequence identified as SEQ ID NO:X..."Total NT Seq." refers to the total number of nucleotides in the contig identified by "Gene No." The deposited clone may contain all or most of these sequences, reflected by the nucleotide position indicated as "5' NT of Clone Seq." and the "3' NT of Clone Seq." of SEQ ID NO:X. The nucleotide position of SEQ ID NO:X of the putative start codon (methionine) is identified as "5' NT of Start Codon." Similarly , the nucleotide position of SEQ ID NO:X of the predicted signal sequence is identified as '5' NT of First AA of Signal Pep.'"

Action is that the specification "...does not provide...adequate written description...that the protein has been expressed by any cell comprising said vector." Applicants respectfully disagree. The specification discloses in Table 1 that the open reading frame (ORF) of the HRDFB85 cDNA encompasses the coding sequence of the SEQ ID NO:344 polypeptide, which has a predicted signal sequence of 21 amino acids. *See* page 467, row 2. Moreover, the specification states that the gene encoded by SEQ ID NO:106 and the HRDFB85 cDNA, "...is homologous to the Clostridium perfringens enterotoxin (CPE) receptor gene product and shares sequence homology with a human ORF specific to prostate and a glycoprotein specific to oligodendrocytes, both of which are tissue specific proteins. See e.g., Katahira et al. J Cell Biol. 136(6):1239-1247 (1997). PMID: 9087440; UI: 97242441." *See* specification page 222, lines 18-22. In view of the fact that the HRDBF85 cDNA encodes a predicted secreted polypeptide that is homologous to several other known polypeptides, Applicants respectfully submit that one of ordinary skill in the art would agree that upon transfection of the HRDFB85 cDNA into a host cell, the predicted open reading frame will express a polypeptide.

IV. Rejections under 35 U.S.C. § 112 (enablement)

Claims 19 and 24-45 have been rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. As a preliminary matter claims 19, 27, 28, 38 and 39 have been canceled, therefore, the rejection with respect to these claims is moot. With regard to claims 24-26, 29-37 and 40-45, the Examiner states the following, "As presented above, the specification does not reasonably suggest that applicant was in possession of the requisite knowledge of normal and disease-indicating levels of the protein corresponding to SEQ ID NO: 344, and the specification does not reasonably suggest that applicant was in possession of the requisite reagents, e.g., antibodies or fragments of same, and the various labeled embodiments." *See* present Office Action, page 9, part 16. Applicants respectfully disagree and traverse.

As a preliminary matter, Applicants submit that in order to enable the claimed method as required by 35 U.S.C. § 112, the specification need only enable a person of ordinary skill in the art to use the claimed method from the disclosure in the patent coupled with information known in the art, without undue experimentation. *United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988). The *United States v. Telectronics, Inc.* case is cited in the M.P.E.P, 8th Edition, revision 2, §2164.01, page 2100-

185, which continues, "[a] patent need not teach, and preferably omits what is well known in the art." *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).

With regard to the notion that "...the specification does not reasonably suggest that applicant was in the possession of the requisite knowledge of normal and disease-indicating levels of the protein corresponding to SEQ ID NO: 344," Applicants point out that the specification teaches that SEQ ID NO:334 is "...expressed primarily in pancreas tumor and ulcerative colitits, and to a lesser extent in several tumors and normal tissues," and that antibodies which bind to polypeptides of the invention can be used to detect aberrant expression of the polypeptide of interest, and thereby diagnose a particular disorder, *e.g.*, "[w]ith respect to cancer, the presence of a relatively high amount of transcript in biopsied tissue from an individual may indicate a predisposition for the development of the disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms..." *See* specification page 223, lines 14-30 and the paragraph bridging pages 545-546. Moreover, as of Applicants' effective priority date, it was a routine practice for individuals of ordinary skill in the art to determine if polypeptides known to be expressed in tumor tissue can be used to distinguish tumor tissue from normal tissue. For example, Lyubsky, *et al.*, evaluated the ability of antibody to B72.3 (an antigen known to be expressed by many types of carcinomas and known to be present in the serum of individuals with pancreatic cancer), to distinguish cancerous and non-cancerous pancreatic samples. *See* Exhibit A, page 160, left column. To carry out this study, biopsied pancreatic tissues (fixed tissues) and pancreatic cells (cytologic aspirates) from patients with confirmed pancreatic cancer, and from healthy patients were obtained from a hospital and stained with anti-B72.3 antibody. *See* Exhibit A, Materials and Methods, pages 160-161. Lyubsky, *et al.*, found "[t]he selective expression of cell surface TAG-72 antigen in primary and metastatic pancreatic cancer in contrast to weak or no expression in pancreatitis or benign pancreas..." indicating the potential value of anti-TAG-72 antibodies in diagnosing pancreatic cancer. In another illustrative example, Yoshida, *et al.*, used anti-laminin immunohistochemistry to stain biopsied liver tissue from patients with confirmed liver cancer and from healthy patients to show that, "...laminin expression is present only in carcinomatous liver tissue and is useful for distinguishing hepatocellular carcinoma from normal or regenerative hepatic tissue." *See*

Exhibit B, pages 70, left column, second paragraph, and page 75, right column, last paragraph of the discussion.

Thus, in light of the fact that (1) Applicants' specification teaches the use of antibodies against SEQ ID NO:344 to detect aberrant expression of SEQ ID NO:344 in pancreatic tumor versus normal pancreatic tissue, and (2) Exhibits A and B show that given the knowledge that a particular protein is predominately expressed in a particular cancer, it was routine for one of ordinary skill in the cancer diagnostic art to compare expression in cancerous tissue with expression in normal tissue, Applicants respectfully submit that the skilled artisan would have been adequately enabled to use antibodies against SEQ ID NO:344 to detect aberrant expression of the SEQ ID NO:344 polypeptide in pancreatic tumor versus non-cancerous pancreatic tissue without undue experimentation.

It has also been alleged that the specification, "...does not reasonably suggest that applicant was in possession of the requisite reagents, e.g., antibodies or fragments of same, and the various labeled embodiments." *See* Office Action, page 9, part 16. As put forth in Applicant's written description response above, the disclosure of the present specification is sufficient to enable one of ordinary skill in the art to make antibodies against SEQ ID NO:344. Moreover, the use of these antibodies to assay SEQ ID NO:344 expression in pancreatic tissue, for example, using immunohistochemical methods, is also enabled by the present specification, for example at page 546, lines 9-12, which states, "[a]ntibodies of the invention can be used to assay protein levels in a biological sample using classical immunohistological methods known to those of skill in the art (e.g., see Jalkanen, et al., J. Cell. Biol. 101:976-985 (1985); Jalkanen, et al., J. Cell . Biol. 105:3087-3096 (1987))."

The Examiner further alleges that the situation at hand is analogous to that in *Genentech v. Novo Nordisk A/S* 42 USPQ2d 100, (CAFC 1997) from which the Examiner emphasized, "...when there is no disclosure of any specific starting material or of any of the conditions under which a process can be carried out, undue experimentation is required; there is a failure to meet the enablement requirement that cannot be rectified by asserting that all the disclosure related to the process is within the skill of the art." *See* Present Office Action, page 10, part 17. Applicants respectfully disagree.

Applicants submit that the present situation differs from *Genentech v. Novo Nordisk A/S*, in that the present specification fully enables the production of the "starting materials" referred to above, *i.e.*, antibodies that bind a polypeptide (SEQ ID NO:344) which is "expressed primarily in pancreatic tumor, ulcerative colitis and to a lesser extent in several

tumors and normal tissues." Furthermore, as described above, one of ordinary skill in the art, as of Applicants' effective priority date, upon reading the present specification coupled with the high level of skill in the antibody based cancer diagnostic art, would be able to use the claimed method to detect aberrant expression of the SEQ ID NO:344 polypeptide in pancreatic tissue versus non-cancerous pancreatic tissue without undue experimentation.

In light of the reasoning given above, Applicants respectfully request that rejection of claims 24-26, 29-37 and 40-45, under 35 U.S.C. § 112, first paragraph, lack of enablement, be reconsidered and withdrawn.

Conclusion

Applicants respectfully request that the above-made amendments and remarks be entered and made of record in the file history of the instant application. The Examiner is invited to call the undersigned at the phone number provided below if any further action by Applicant would expedite the examination of this application.

If there are any fees due in connection with the filing of this paper, please charge the fees to our Deposit Account No. 08-3425.

Respectfully submitted,

Date: 4/20/2005

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A Tumor-Associated Antigen in Carcinoma of the Pancreas Defined by Monoclonal Antibody B72.3

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A retrospective analysis of 25 primary adenocarcinomas of the pancreas, 16 metastatic pancreatic tumors, 8 cases of chronic pancreatitis, and 3 adult normal pancreas was performed to ascertain the reactivity of monoclonal antibody (MAb) B72.3 to malignant and nonneoplastic pancreatic lesions. Formalin-fixed, paraffin-embedded sections of pancreas were evaluated by immunohistochemical techniques (avidin–biotin–peroxidase complex [ABC] method). Twenty-one of 25 malignant primary tumors were reactive, and all 16 metastatic sites expressed the B72.3 antigen. In contrast, all cases of pancreatitis and normal pancreas were either weakly reactive or nonreactive. Ten malignant and two benign pancreatic fine-needle aspirates provided results similar to those seen with fixed tissues. Because MAb B72.3 has selective reactivity for primary and metastatic pancreatic cancer, it may be of value as a diagnostic adjunct in cytologic examination or for radioimmunodetection of regional and/or distant metastases of adenocarcinoma of the pancreas. (Key words: Monoclonal antibody; Immunohistochemistry; Pancreatic cancer) Am J Clin Pathol 1988;89:160–167

IN PREVIOUS immunohistochemical studies with formalin-fixed, paraffin-embedded tissues, monoclonal antibody (MAb) B72.3 has exhibited reactivity with a wide spectrum of carcinomas, including 94% of colonic adenocarcinomas, 84% of invasive ductal carcinomas of the breast, 96% of non–small cell lung carcinomas, and 100% of common epithelial ovarian carcinomas, but very limited reactivity with the respective normal tissues.[1] Its selectivity has been further supported by its failure to react with nonepithelial malignancies. The antigen to which MAb B72.3 reacts is a mucin-like glycoprotein (TAG-72) with a molecular weight of approximately 1×10^6 daltons.[11] It is expressed in several endodermally derived organs in the fetus but not in fetal pancreas.[21] In gastrointestinal malignancies, including pancreatic cancer, shed antigen is present in the serum.[18] Our purpose was to determine if MAb B72.3 demonstrated adequate sensitivity and specificity in pancreatic cancer to merit its use in a trial as an adjunct in cytologic examination and as a tool for radioimmunodetection of pancreatic cancer.

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Materials and Methods

Monoclonal Antibody

MAb B72.3 was generated by hybridoma culture obtained after the fusion of splenic lymphocytes from mice immunized with a membrane-enriched fraction of a human breast carcinoma metastasis to the liver, with a non–immunoglobulin-secreting myeloma cell line.[5,22] MAb B72.3 was obtained from Dr. David Colcher at the Laboratory of Tumor Immunology and Biology, National Cancer Institute, Bethesda, Maryland. The purification of the MAb B72.3 has been described previously.[22]

Case Material

Paraffin-embedded blocks of formalin-fixed tissue from the Veterans Administration Medical Center, Northport, and Winthrop University Hospital, Mineola, from the period of 1973–1986 were used for this study. The material obtained for this study included the following: 25 primary adenocarcinomas of the pancreas, 16 metastatic pancreatic tumors from six patients, 8 specimens of chronic pancreatitis, and 3 specimens of adult normal pancreas. Nine of the 11 benign cases were from surgical specimens. In the autopsy material, only cases with minimal autolysis were selected. Hematoxylin and eosin sections were reviewed to confirm the histologic diagnosis.

Immunoperoxidase Assay

Fixed Tissues. From formalin-fixed paraffin-embedded tissues, 5-μm tissue sections were mounted on gelatin-coated glass slides and heated for one hour at 60 °C. A modification of the avidin–biotin–peroxidase com-

plex (Vectastain® ABC Kit, Vector Laboratories, Burlingame, CA) was used as described previously.[10] The sections were incubated overnight at 4 °C with the use of MAb B72.3 (the primary antibody) in a final concentration of 60 μg/mL. MOPC-21® (Litton Bionetics, Kensington, MD) was used as an irrelevant IgG_1 isotype negative control at an identical concentration to the primary antibody.

A semiquantitative method was used to score the slides. Only staining of strong (2+) or moderate (1+) intensity with definite brown color was considered positive. A weak stain or faint blush (±) and no staining were considered negative results. Additionally, the percentage of malignant cells reactive with MAb B72.3 was estimated by scanning the entire section and counting stained tumor cells *versus* unstained cells in representative high-power fields.

Cytologic Aspirates. Papanicolaou-stained aspirates (ten malignant and two benign) from the years 1982–1985 were used for the study. Coverslips were soaked off in xylene, and the slides were then stained in a similar fashion to fixed tissues with two exceptions: (1) the concentration of primary antibody employed was 40 μg/mL, and slides were incubated with primary antibody overnight at 4 °C; (2) slides were treated with hematoxylin for only 5 seconds to avoid overstaining of nuclei.

Results

Most of the tumors[24] were well or moderately well differentiated, only one case being poorly differentiated. We first determined staining intensity and patterns of staining in malignant and benign pancreatic cases. Although an initial titration study indicated reactivity of malignant tumors at 20 μg/mL, we selected 60 μg/mL as the concentration because we were trying to determine maximal reactivity in the absence of background. Twenty-one of 25 malignant cases stained with strong or moderate intensity (Fig. 1). Between 10 and 100% of the cells expressed the B72.3 antigen (Fig. 2). The staining pattern was predominantly cytoplasmic, although apical and membranous staining were also seen. Heterogeneity of staining was observed in most cases (Fig. 3*A*). We had too few poorly differentiated cases to determine if there was a difference in antigen expression between well- or moderately well-differentiated and poorly differentiated carcinomas.

On the other hand, all cases of pancreatitis and normal pancreas were weakly reactive (+/−) or had negative results (Fig. 3*B*). Benign ductal cells showed faint apical staining. In two cases of chronic pancreatitis weak cytoplasmic staining of acini was seen, and in a single case weak staining of islet cells was observed.

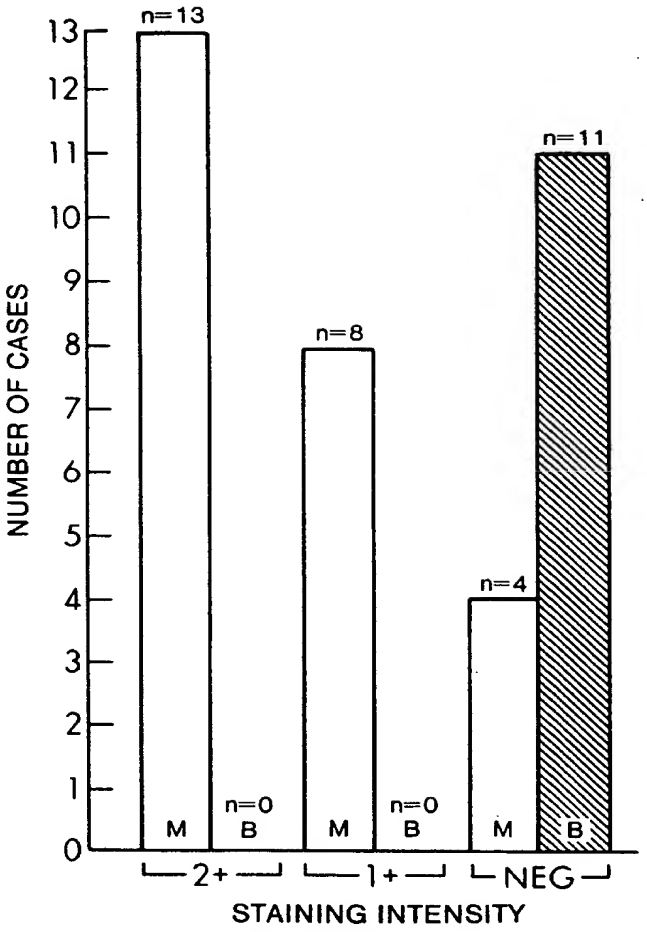

FIG. 1. Intensity of reactivity. MAb B72.3 was used at a concentration of 60 μg/mL in an overnight assay. Positive cells were graded in intensity of reactivity as follows: ++ (strong), + (moderate), +/− (weak), or 0 (negative). For purposes of scoring, +/− staining was considered negative; M = malignant; B = benign.

Table 1 compares the reactivity of primary tumors with that seen in immunoperoxidase assays of the metastatic sites. All 16 metastases of pancreatic cancer expressed the antigen. We observed that decalcification procedures do not decrease the expression of the antigen in bone marrow. Figures 4A–C demonstrated the reactivity of lymph node, bone marrow, and liver metastases. Staining patterns were similar to those observed in primary tumors.

In all cases of tumor there was a chronic inflammatory and desmoplastic reaction in surrounding benign tissue (tumor-associated pancreatitis [TAP]). Weak apical luminal staining of the duct epithelium was occasionally observed in the areas of TAP. This phenomenon may result from shedding of tumor antigen reactive with MAb B72.3.[4]

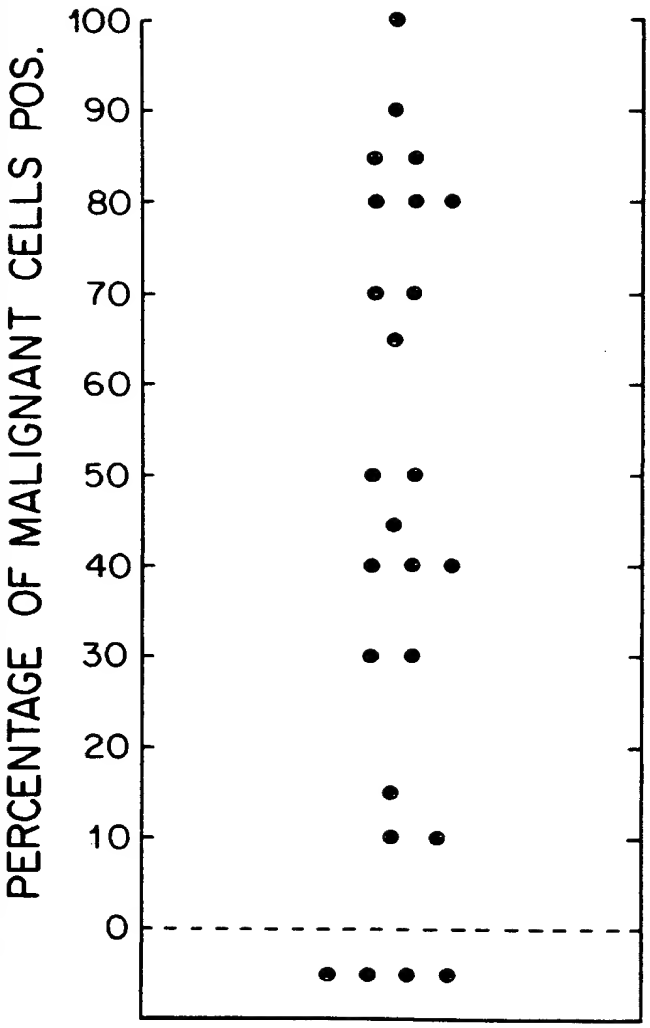

FIG. 2. Percentage tumor cells positive by immunoperoxidase assay. A semiquantitative approach was used for scoring. After scanning of the entire slide, representative high-power fields were used to further estimate stained *versus* unstained tumor cells.

A Papanicolaou smear obtained from a computed tomography (CT)–directed fine-needle aspiration biopsy of a pancreatic adenocarcinoma was superstained with MAb B72.3. Reactivity was noted in malignant duct cells (Fig. 5). In view of this finding, an additional nine malignant and two benign cytologic cases were stained in similar fashion. All malignant cases reacted with moderate to strong intensity, with a range of 25–75% of tumor cells staining positively. One case originally diagnosed as atypia by cytologic examination stained strongly with the MAb. The biopsy confirmed the presence of a well-differentiated adenocarcinoma.

Discussion

The selective expression of the cell surface TAG-72 antigen in primary and metastatic pancreatic cancer in contrast to weak or no expression in pancreatitis or benign pancreas suggests that MAb B72.3 may be of value as a diagnostic adjunct in cytologic aspirates of pancreatic cancer as well as radioimmunodetection.

Other MAbs reactive to pancreatic cancer have been generated with the use of human pancreatic cancer cell lines as the immunogen. The DU-PAN antibodies react with either normal pancreas or fetal pancreas. DU-PAN-2, an IgM, reacts with carcinomas outside of the gastrointestinal tract but to a lesser degree than MAb B72.3. DU-PAN-1 and DU-PAN-3 are more pancreas specific.[1,12,16] MAb AR 1-28 is reactive to a membrane-associated antigen of molecular weight of 200,000 daltons. It reacted to 23 of 27 pancreatic cancers tested, demonstrating a predominant apical staining pattern in well-differentiated and moderately well-differentiated tumors. It was less reactive to poorly differentiated cancers.[3]

MAB AR 1-28 did not react with normal pancreatic, ductal, and islet cells.[2] Its reactivity to metastatic pancreatic cancer and application to clinical aspiration cytologic examination were not studied.

The early diagnosis of pancreatic carcinoma can be made by aspiration cytologic examination with diagnostic accuracy between 60 and 90% among experienced cytopathologists.[7–9,15,19,20] The major problems encountered are as follows: (1) very well differentiated adenocarcinomas are difficult to distinguish from atypical and reactive ductal cells as seen in severe pancreatitis; (2) some pancreatic carcinomas have a marked scirrhous component; the malignant cells are not released from their fibrous matrix, with resultant sparse cells on the slide.

Although we tested it on only 12 pancreatic aspirates, we were encouraged by seeing no staining of benign ductal cells, similar to what is observed on fixed tissue sections. In addition, in 1 of 10 malignant cases it proved to be a useful diagnostic adjunct to the cytopathologist. Other authors have suggested its diagnostic value in adenocarcinoma in pleural effusions[23] and breast aspirates.[14]

FIG. 3. *A* (*upper*). Primary pancreatic cancer. Positive cytoplasmic staining of adenocarcinoma of pancreas (×400). Note heterogeneity of staining. *B* (*lower*). Benign pancreas. Ductal cells from benign pancreas demonstrating absence of reactivity to MAb B72.3 (×400).

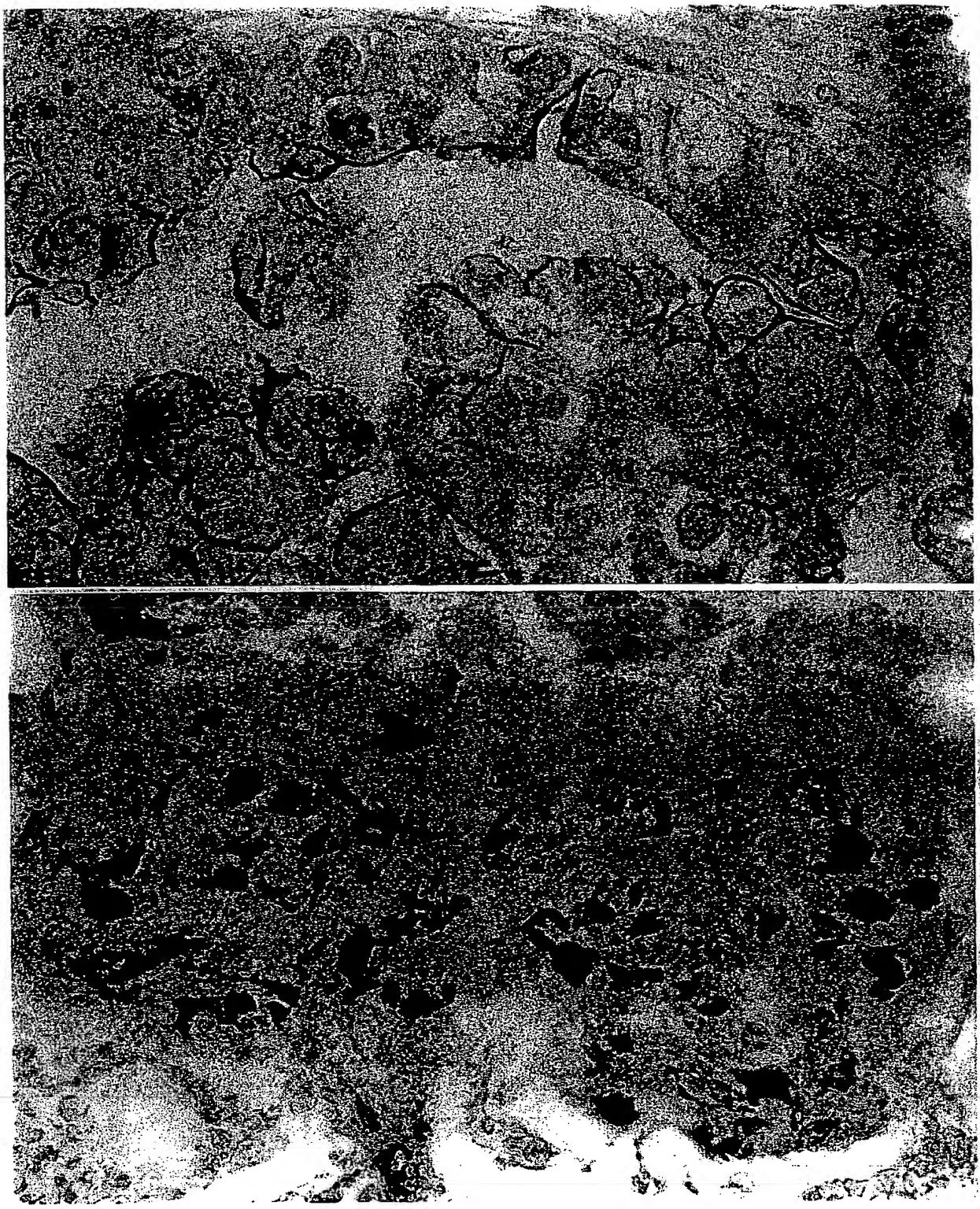

FIG. 4. *A* (*upper*). Regional node metastasis. Strong apical and membranous staining of a regional lymph node metastasis (×400). *B* (*lower*). Bone marrow metastases. Cytoplasmic staining of bone marrow metastasis of pancreatic adenocarcinoma (×400). (*continued.*)

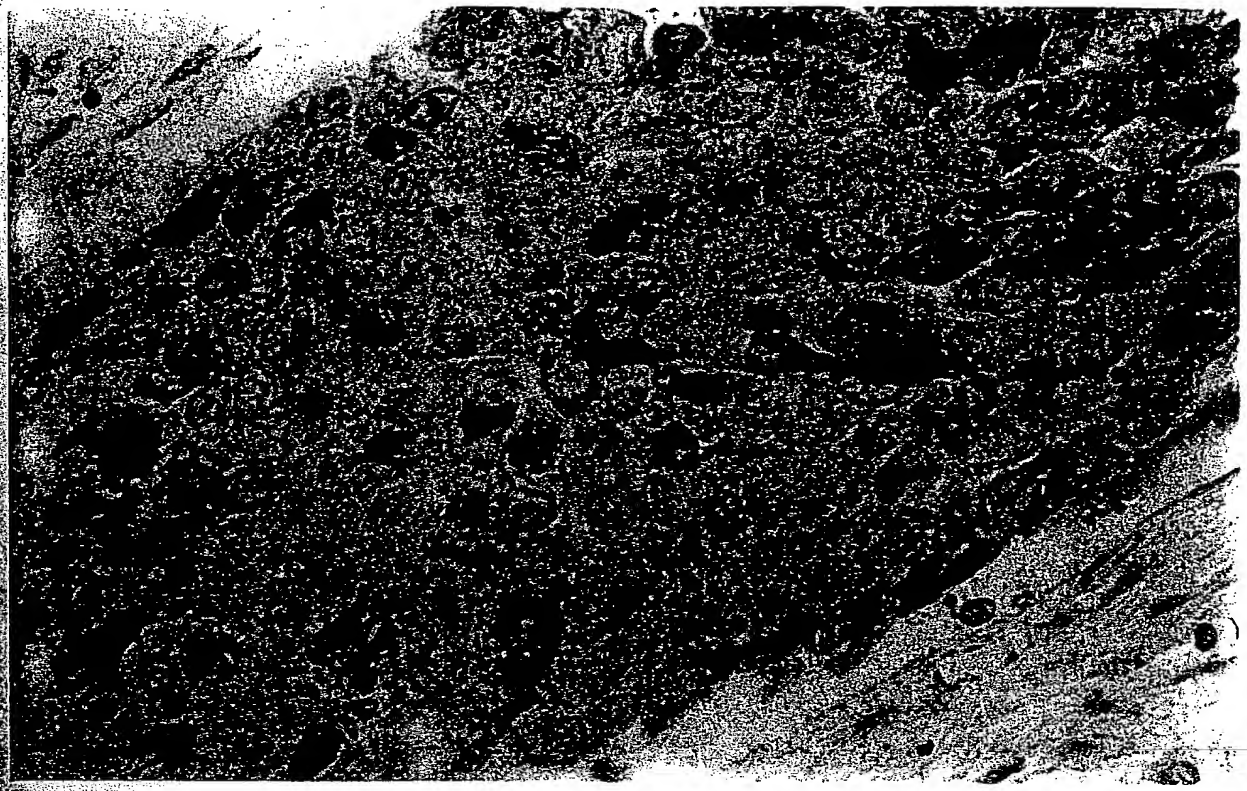

FIG. 4 (*continued*). *C.* Liver metastasis. Heterogeneous cytoplasmic staining of tumor embolus in portal venule (×400).

Assessment of extent of disease in pancreatic cancer requires several diagnostic modalities (ultrasound, CT scan, endoscopic retrograde cholangiopancreatography),[6,17] but at present the extent of regional disease and even liver metastases is often underestimated before operation. The encouraging aspect of our study is that all regional and distant metastases express the TAG-72 antigen to at least an equivalent degree as expressed in the primary tumor. In addition, if we include the aspirated material, 28 of 35 (80%) primary cancers had at least 25% of tumor cells staining positively. Initial radioimmunodetection studies in patients with colon

Table 1. Reactivity of Primary Tumors and Metastatic Sites

Primary*	Metastases†							
1. 80 / +	Lymph node 90 / +	Bone 80 / ++	Lung 95 / ++	Liver 85 / +	Adrenal 65 / +	Small intestine 95 / ++	Colon 100 / ++	Brain 65 / ++
2. 10 / ++				Liver 5 / +				
3. 40 / ++		Bone 50 / ++	Lung 80 / +	Liver 40 / ++				
4. 85 / ++		Bone 90 / ++			Adrenal 90 / ++			
5. 100 / ++	Lymph node 100 / ++							
6. 85 / ++		Bone 100 / ++						

* Primary adenocarcinomas (1–6). Top figure: Percentage of cells staining positively. Bottom figure: Intensity of staining. Scoring method has been described in text.

† Metastatic sites. Represented in an identical fashion to the primary sites.

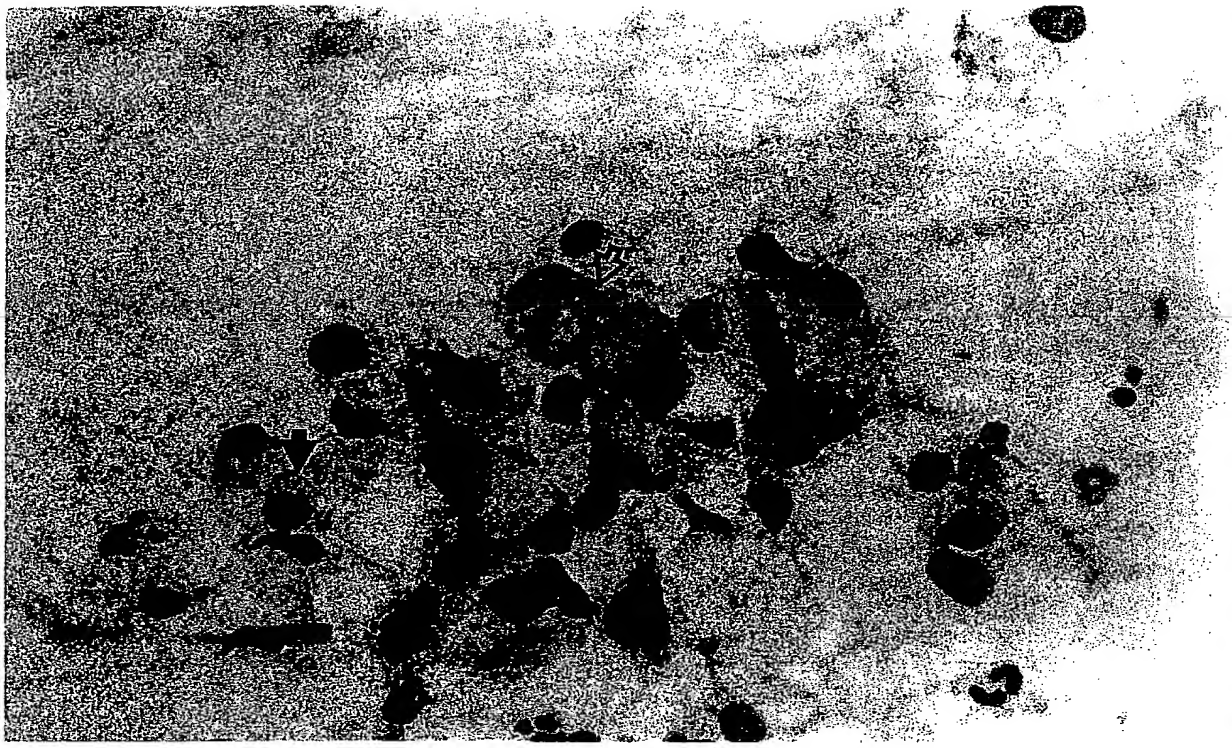

FIG. 5. Aspirate of pancreatic mass. *Open arrow* indicates positive cytoplasmic staining of malignant cells (×400). *Closed arrow* constrasts an unstained benign cell.

cancer with the use of ^{131}I B72.3 to detect metastases have demonstrated good tumor-to-blood and tumor-to-normal tissue ratios, with imaging possible in more than 50% of patients.[4] Larson and associates have demonstrated the ability to image melanoma with radiolabeled antitumor antibodies.[13] Such studies should serve as an impetus for clinical trials using radiolabeled B72.3 F(ab')$_2$ to detect spread of pancreatic cancer before operation.

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Expression of Laminin in Hepatocellular Carcinoma: An Adjunct for Its Histological Diagnosis

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In general, hepatocytes lack basement membrane structures and therefore no laminin expression is seen around hepatic cords. To determine whether or not laminin expression appears when hepatic tissue becomes carcinomatous, we carried out immunohistochemical staining of hepatic tissues excised surgically from 35 patients with hepatocellular carcinoma, 18 with metastatic colon carcinoma, two with adenomatous hyperplasia, and 10 without any nodular lesions. Among the various conditions of hepatic tissue, laminin expression was detected only in hepatocellular carcinoma with 86% positivity. The result was not dependent on the degree of differentiation. Therefore, it was confirmed that immunohistochemical detection of laminin provides a useful adjunct for the diagnosis of hepatocellular carcinoma, and this was verified by a study using needle biopsy samples. In addition, our results suggested that the basement membranes are derived from endothelial cells of either portal veins or hepatic arteries.

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Key words: Immunohistochemistry—Hepatocellular carcinoma—Laminin—Hepatic sinusoid

Introduction

A well differentiated hepatocellular carcinoma (HCC) may histologically resemble normal and/or regenerative liver tissue,[1–3] and it is often difficult to differentiate from the latter conditions, especially in needle biopsy specimens.

Laminin is a component of the extracellular matrix and exists within the basement membrane.[4, 5] In normal liver tissue, well defined basement membranes never surround the hepatic cords, and therefore laminin is not present along the sinusoids.[6, 7] In HCCs, however, laminin may be expressed around tumor cell nests, and is detectable by immunohistochemistry.[8–10] If, indeed, laminin appears only in carcinomatous liver tissue, its detection could be a useful adjunct for histological diagnosis. To verify this possibility, we carried out immunohistochemical staining of laminin in tissues of HCC, liver cirrhosis, and chronic hepatitis as well as non-neoplastic, non-inflammatory liver. Our results suggested that laminin is expressed only in carcinomatous liver tissue and that it is a useful marker for diagnosis of HCC.

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Materials and Methods

For this study, we used hepatic tissues excised surgically from 35 patients with HCC, 18 with metastatic colon cancer in the liver, two with adenomatous hyperplasia and 10 without any nodular lesions (Table I). Then, to determine whether or not the same principle could be applied to needle liver biopsy specimens, and whether detection of laminin would be useful for the diagnosis of HCC, 100 biopsy samples, including 29 samples of HCC, 23 of liver cirrhosis, 41 of chronic hepatitis, and seven of normal liver were utilized (Table II). The diagnosis in each case depended upon not only histological changes but also preoperative biochemical data, the serum levels of tumor markers, and the results of diagnostic imaging, including computed tomographic scans of the liver and echography. For the first study, HCCs were histologically subclassified into well differentiated, moderately differentiated, poorly differentiated and undifferentiated carcinomas according to the classification of The General Rules for the Clinical and Pathological Study of Primary Liver Cancer.[11] Because a single surgically excised nodule of HCC may com-

Table I. Diagnosis of the Resected Samples

Nodular lesions		Diagnosis of nodules		Diagnosis of surrounding tissue		
				normal	CH	LC
(+)	55	HCC	35	0	9	26
		Meta.	18	12	4	2
		AH	2	0	0	2
(−)	10			0	4	6
Total	65		55	12	17	36

HCC, hepatocellular carcinoma; Meta., liver metastasis from colon carcinoma; AH, adenomatous hyperplasia; CH, chronic hepatitis; LC, liver cirrhosis.

Table II. Diagnosis and Expression of Laminin in Needle Samples

Laminin expression	normal	CH	LC	HCC
positive/total	0/7	0/41	0/23	18/29
%	0	0	0	65.5

*, $P<0.025$ (between normal and HCC); †, $P<0.005$ (between CH and HCC, LC and HCC); CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma.

Table III. Laminin Expression in Resected Samples

Laminin	normal	CH	LC	AH	HCC (degree of differentiation)				
					all of HCC	well	mod.	por.	un.
positive/total	0/12	0/17	0/36	0/2	44/50	23/26	17/17	3/5	1/2
%	0	0	0	0	88.0	88.5	100	60	50

*, $P<0.005$ (vs HCC); †, $P<0.025$ (vs HCC); CH, chronic hepatitis; LC, liver cirrhosis; AH, adenomatous hyperplasia; HCC, hepatocellular carcinoma; well, well differentiated; mod., moderately differentiated; por., poorly differentiated; un., undifferentiated.

prise areas which differ in their degree of differentiation, we divided the lesions into areas showing specific degrees of differentiation and counted each area as a single lesion. This gave a total of 26 well differentiated, 17 moderately differentiated, five poorly differentiated and two undifferentiated lesions. We utilized non-tumorous liver tissue samples as controls. Because the liver tissue adjacent to HCC contained cirrhosis in 26 cases and chronic hepatitis in nine, and that adjacent to metastatic colon cancer was normal in 12 cases, showed chronic hepatitis in 4 and cirrhosis in two, the control materials finally included 36 samples of cirrhosis, two of adenomatous hyperplasia, 17 of chronic hepatitis and 12 of normal liver.

All the tissues were fixed in 10% buffered formalin, processed routinely and embedded in paraffin. Four-micrometer-thick sections were obtained for histological examination and stained with hematoxylin-eosin, and some of them were stained immunohistochemically with an anti-laminin antibody. For immunohistochemistry, we used the avidin-biotin-peroxidase complex (ABC) method.[12, 13] Briefly, the sections were deparaffinized and incubated with 0.1% pepsin (Sigma P-6887, Sigma Chemical Co., St. Louis, MO)/0.01 N-HCl at 37°C for 100 min. After washing, they were again incubated with 0.05% pronase (Sigma P-6911) in phosphate-buffered saline at 20°C for 10 min.[14] Intrinsic peroxidase activity was inhibited by 0.3% H_2O_2-methanol, and any non-specific reaction was blocked with normal goat serum. Next, the tissue sections were reacted with a rabbit anti-human laminin antibody (polyclonal; Bio-science Products AG, Emmenbrücke, Switzerland) for 120 min at 20°C and then with an Elite ABC kit (Vector Laboratories Inc., Burlingame, CA). Finally, 3,3'-diaminobenzidine tetrahydrochloride was used as a chromogen, and the sections were counterstained with Carazzi's hematoxylin, dehydrated, and mounted. Dark brown linear deposits along and/or around the hepatic cords were considered to indicate positive result. Positive and substitution controls were stained in parallel with the test materials. The positivity of laminin expression in normal, inflammatory and neoplastic liver tissue was compared and tested by χ^2 analysis.

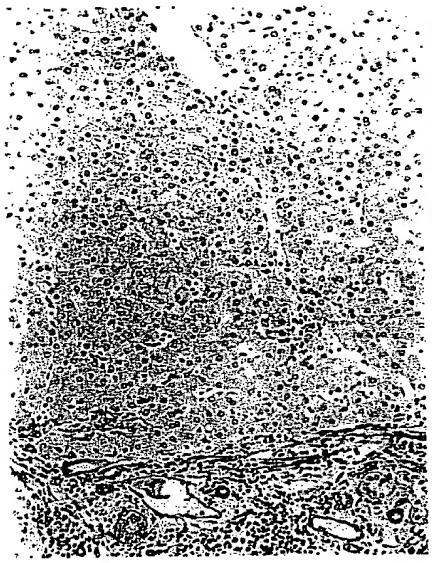
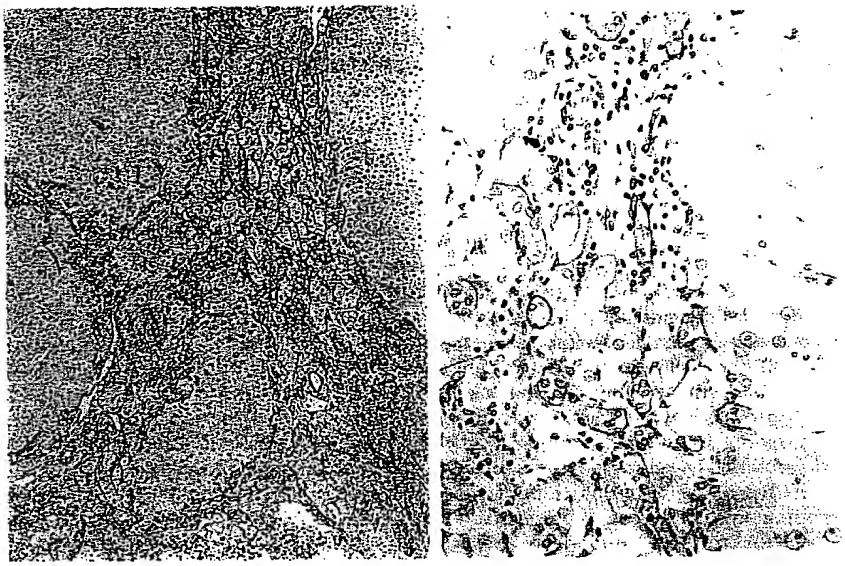
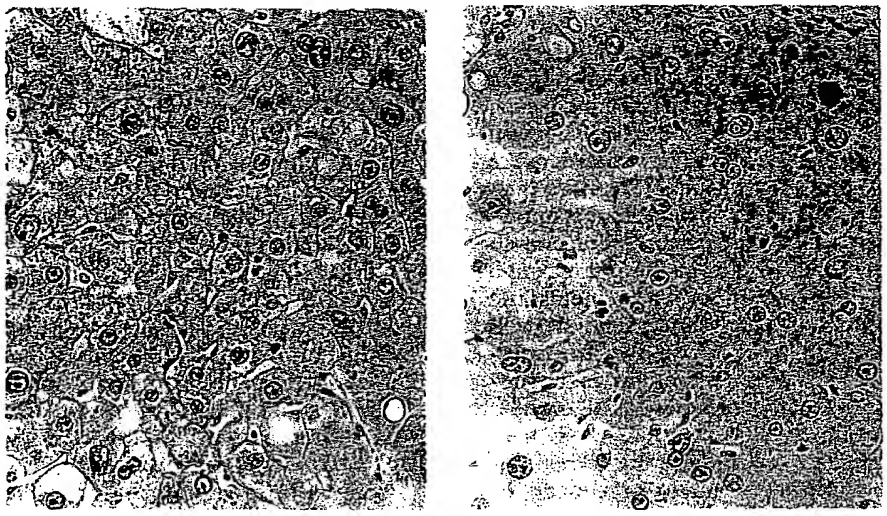


Fig. 1. Immunohistochemistry of laminin in chronic hepatitis. Immunoreactivity is absent in the perisinusoidal space, although there is positive expression of laminin in the basement membrane of vessels and biliary ducts in the portal area.



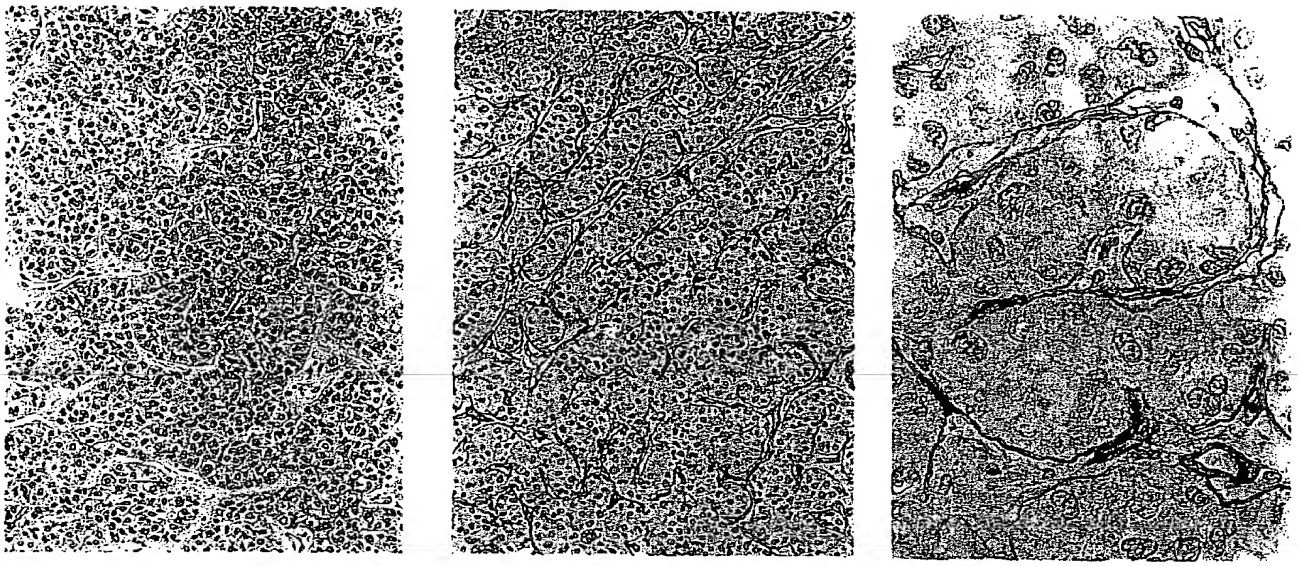
(a) (b)

Fig. 2. Immunohistochemistry of laminin in cirrhotic liver tissue. A linear reaction is seen at the periphery of the pseudolobules. Note that the hepatocytes within the pseudolobules are not stained for laminin.



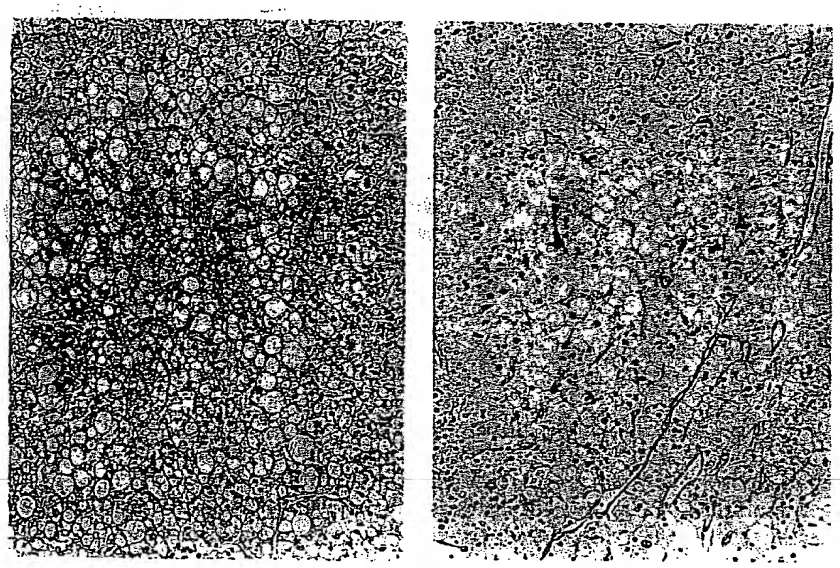
(a) (b)

Fig. 3. Liver cirrhosis. Nuclear atypism is prominent in some lesions, but laminin reactivity is absent (3a: HE, 3b: ABC).



(a) (b) (c)

Fig. 4. Immunohistochemistry of laminin in HCC. A linear positive reaction is seen in the perisinusoidal space, surrounding a nest of HCC (4a: HE, 4b-c: ABC).



(a) (b)

Fig. 5. Well differentiated hepatocellular carcinoma. Fatty change and little nuclear atypism would hinder the recognition of this tumor as hepatocellular carcinoma. However, because laminin expression is evident along the sinusoids in places, the tumor can be positively diagnosed (5a: HE, 5b: ABC)

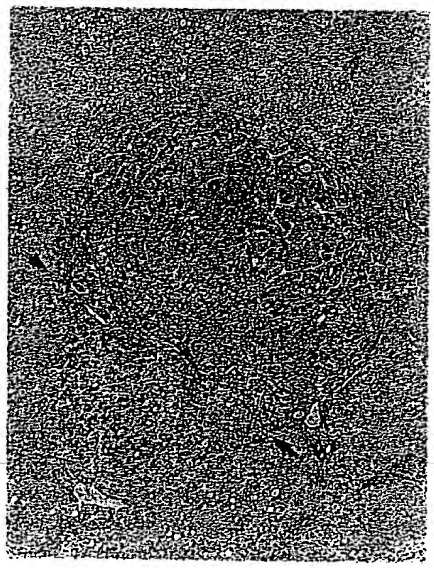
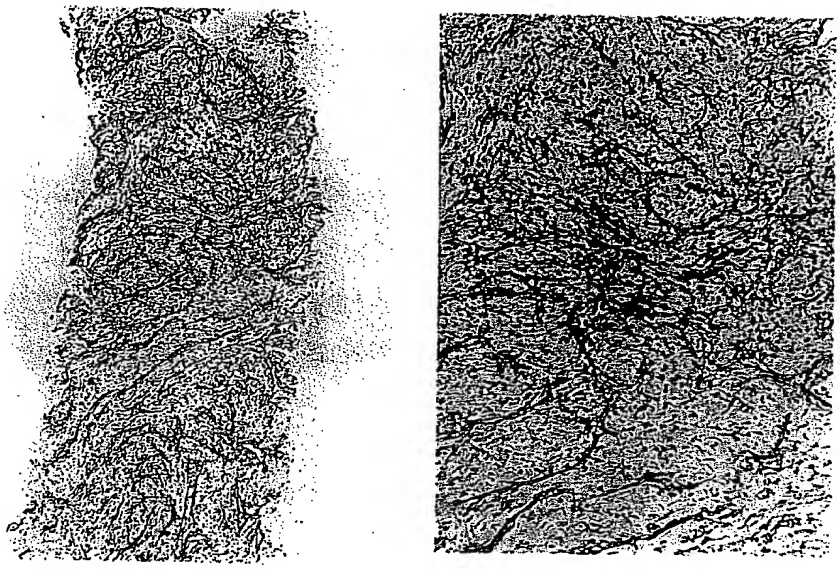


Fig. 6. Immunohistochemistry of laminin in a small HCC nodule. Immunopositive materials are seen near the portal area (arrows) but not in the central.



(a) (b)

Fig. 7. Immunohistochemistry of laminin in a biopsy sample of HCC. The pattern of immunoreaction is similar to that in resected samples. The cells are poorly differentiated, spindly carcinoma cells.

Results

Among the liver tissues taken by excision, laminin reactivity was detected only in HCCs (Table III). In normal and chronically inflamed tissues, no laminin expression was seen within the hepatic lobules (Fig. 1). Cholangioles and blood vessels in the portal areas, however, were surrounded by linear immunoreactive deposits, as were colonic adenocarcinomas. Cirrhotic liver showed a distribution pattern essentially similar to the normal one, except for a few cases where linear reaction products were present at the extreme periphery of the pseudolobules, surrounding hepatocytes contiguous with the portal venous or arterial system (Fig. 2a, b). In the latter cases, cholangioles merging into hepatic cords were also immunopositive. However, the remaining hepatocytes in the pseudolobules were consistently negative (Fig. 3a, b). Areas of adenomatous hyperplasia were also negative for laminin. In contrast, laminin was positive in 30 out of 35 cases (86%) of HCC. Generally, linear laminin-positive deposits were located between the sinusoidal endothelium and hepatoma cells, surrounding nests of carcinomatous hepatocytes (Fig. 4a, b, c). When laminin was stained, it was distributed evenly within the tumor mass, irrespective of the location. When the degree of differentiation was taken into account, 23 of 26 well differentiated (Fig. 5a, b), 17 of 17 moderately differentiated, 3 of 5 poorly differentiated, and 1 of 2 undifferentiated lesions were positive for laminin (Table III). There were no significant differences in positivity among carcinomas with different degrees of differentiation or architectural patterns ($P>0.05$). On the other hand, in some tumors, particularly small ones less than 1 mm in diameter, positive reactions were seen near portal areas but not in the central portion (Fig. 6). In addition, large trabeculae of HCC exhibited widely separated immunopositive basement membranes.

In biopsy samples, the results were similar to those for resected materials with regard to positivity (Table II) and distribution pattern (Fig. 7a, b). Overall immunopositivity in HCCs was 62%. All the other tissues were completely negative for laminin expression.

Statistical analysis indicated a significant difference in immunopositivity between benign hepatic tissues and hepatocellular carcinoma (Tables II and III).

Discussion

It is generally said that the basement membrane does not exist in normal hepatic lobules. This was

first noted in electron microscopy studies, which found that electron-dense basement membrane materials were absent between hepatocytes and sinusoidal endothelial cells, [6, 15–17] and then in immunohistochemical studies where hepatic cords were found to lack surrounding laminin,[7, 10] despite the presence of type IV collagen, another component of the basement membrane. This unique structure, as well as fenestration of endothelial cells, is considered to facilitate the transport of a variety of substances between the blood and hepatocytes. However, it has been demonstrated ultrastructurally that electron-dense materials of the basement membrane may appear in association with liver fibrosis.[7, 10] Interestingly, immunohistochemical expression of laminin never appears in the central portion of pseudolobules,[7, 10] but at their extreme periphery. In HCCs, laminin expression develops beneath the endothelial cells surrounding nests of carcinoma cells,[8, 18] as shown in this study.

There are two implications of the present data: (1) laminin expression appears in HCC and (2) laminin expression or the formation of basement membrane may be carried out by endothelial cells of the portal venous or arterial system. In our study with resected materials, the presence of laminin immunoreactivity was detected only in cases of HCC, implying that laminin expression in hepatocytic lesions was specific for HCCs. This feature may be important in surgical pathology because the immunohistochemical presence of laminin might be an excellent marker for diagnosis of HCC in cases where it is histologically difficult to differentiate from regenerated hepatocytes, particularly in small needle biopsy samples. However, not all HCCs expressed laminin, indicating that there is a limitation to its usefulness. Therefore, we undertook a similar study using needle biopsy samples to determine how accurately the results could be used as an indication of malignancy. In contrast to the overall positivity of 86% in resected samples, that of the needle biopsy samples was 62%. This difference between the two procedures may be due to the amount of tissue taken in each. In HCCs with thin trabecular structures, laminin deposits were located close to each other, but those of thick trabecular structures were rather widely separated. In other words, the amount of laminin may vary according to the thickness of the hepatoma trabeculae, and in thicker trabeculae the positivity becomes less than that in thin ones. This is because in carcinomas with thick trabecular structures, the needle tract may not pass through many portions where laminin is located. Absence of laminin in the central portion of a minute hepatoma does not hinder the usefulness of this procedure for detection of HCC, because a 1-mm hepatoma nodule would not be detected clinically or biopsied intentionally. Another feature necessitating caution is the presence of laminin exprerssion at the extreme periphery of pseudolobules in cirrhotic liver, and so possible over-reading should be avoided. However, bearing these possible pitfalls in mind, we conclude that detection of laminin in the diagnosis of HCC is useful.

The origin of laminin in HCC still remains to be elucidated. The expression of laminin may result from sinusoidal capillarization in hepatocellular carcinoma. However, our study shows there are several reasons to believe that laminin may be produced by endothelial cells of either the portal veins or hepatic arteries, although it cannot be completely ruled out that carcinomatous hepatocytes can produce these laminins. First, laminin was expressed in the outermost portions of pseudolobules in cirrhotic liver, which are connected to blood vessels of portal areas, particularly portal veins. Second, HCC is believed to be nourished by hepatic arteries and portal veins.[9, 19–21] Third, small nodules of HCC in our study showed an uneven distribution of laminin, the expression being stronger in zones near portal areas and absent near the central vein. From the above findings we can speculate as follows. HCC cells may show active proliferation, but they may not necessarily have the same functions as normal hepatocytes. Therefore they may require more nutrients and oxygen, which may be supplied by either the portal veins or hepatic arteries. For this reason, as the tumor grows, endothelial cells from these vessels must extend or replace sinusoidal structures with basement membrane material. To validate this hypothesis, further studies will, of course, be necessary.

In summary, our study has clearly demonstrated that laminin expression is present only in carcinomatous liver tissue and is useful for distinguishing HCC from normal or regenerative hepatic tissue. Therefore, this feature is expected to be a useful adjunct for histological diagnosis in difficult cases.

Acknowledgments

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